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ISSN 0792 - 156X

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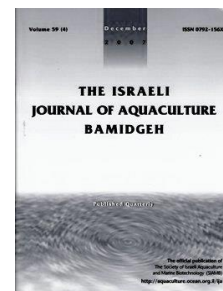
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Hybrid Origin of the Thai-Chitralada Tilapia Strain Using Dna Barcoding and Microsatellite Analysis

Curzon A.Y.^{1,2}, Shirak A.², Dor L.^{1,2}, Zak T.³, Benet-Perelberg A.³, Seroussi E.² and M. Ron*²

¹Robert H. Smith Faculty of Agriculture, Food and Environment, Hebrew University of Jerusalem, Rehovot 76100, Israel

²Institute of Animal Science, Agricultural Research Organization, Bet Dagan 50250, Israel

³Dor Research Station, Fisheries and Aquaculture Department, Ministry of Agriculture and Rural development, Israel

Keywords: mitochondrial DNA; microsatellites; phylogenetic tree; Thai Chitralada tilapia; *Oreochromis niloticus*

Abstract

The Thai-Chitralada strain originates from Egypt and was transferred to Japan. From there a stock of 50 fish were introduced to the Royal Chitralada Palace in Thailand. The commercially cultured strain of Nile tilapia *O. niloticus* in Thailand, Thai-Chitralada, possesses desirable traits for aquaculture, such as high growth rate integrated with reasonable fecundity and excellent performance in Asian countries. In 2010 a few dozen Thai-Chitralada fish were introduced to Israel from Thailand. The objective of this study is to trace the origin of Thai-Chitralada using DNA barcoding and microsatellite genetic markers. *Cytochrome Oxidase I* and D-loop sequences of 19 Thai-Chitralada fish clustered into three groups comprising 6, 7, and 6 individuals were homologous to the consensus sequences of *O. aureus*, *O. niloticus*, and *O. mossambicus*, respectively. The allele ranges for microsatellites UNH168 and G7A exclude *O. niloticus* (Ghana) as potential contributor to the Thai-Chitralada strain. Genotyping for three microsatellites indicate overlap of alleles between Thai-Chitralada and *O. niloticus* (Egypt) and *O. aureus*, while *O. mossambicus* was not tested. Thus, our data based on mitochondrial and genomic analyses demonstrate that *O. aureus*, *O. niloticus* (Egypt), and *O. mossambicus* contributed to the formation of Thai-Chitralada. Thai-Chitralada can be used as a genetic resource for selection and adaptation to different geographical regions because of its diverse genetic background and desirable traits.

Introduction

Tilapias are divided into three genera: *Oreochromis*, *Sarotherodon*, and *Tilapia*, while interspecific hybrids in each genus, and between *Oreochromis* and *Sarotherodon* species are viable. The genus *Oreochromis* contains more than 30 species and is one of the most cultivated fish. The main drive for the hybridization of tilapia species was to produce all male populations (Hickling, 1960; Mires, 1969) which resulted in contact between previously geographically isolated species (Elder and Garrod, 1961). Investigation has indicated the existence of a genetically based reproductive barrier between related *Oreochromis* species (Shirak et al., 2018). Nile tilapia *O. niloticus*, was transferred from its origin in Africa to natural and artificial water bodies worldwide (Trewavas, 1983). In the last two decades, breeding programmes achieved significant progress in tilapia, which are used widely in aquaculture and in the developing world (Mair et al., 2004). Tens of mitochondrial sequences of *Cytochrome Oxidase I (COI)* and D-loop for each of the main species e.g., *O. niloticus*, *O. mossambicus*, and *O. aureus* have been deposited in the Barcoding of Life database (FISH_BOLD; Ward et al., 2009) and/or GenBank, respectively. Rapid growth of the FISH-BOL database has been facilitated by a universal primer cocktail that is capable of amplifying the *COI* fragment in more than 93% of fish species (Ivanova et al., 2007). More than 20,500 of the estimated 32,000 fish species (64.1%) have been barcoded (http://boldsystems.org/index.php/TaxBrowser_Home). However, mitochondrial DNA sequences could be confounded due to hybridization between *Oreochromis* species (Elder and Garrod 1961; Firmat et al., 2013; Ovenden et al., 2014).

The Thai-Chitralada strain originates from Egypt and was transferred to Japan, from which a stock of 50 fish had been introduced to the Royal Chitralada Palace in Thailand in 1965 (Mair et al., 2004). The commercially cultured strain of *O. niloticus* in Thailand, Thai-Chitralada, possess desirable traits for aquaculture, such as high growth rate integrated with reasonable fecundity, tolerance to high water salinity, and excellent performance in Asian countries (Macaranas et al., 1997). Thus, the objective of this study is to trace the origin of Thai-Chitralada using DNA barcoding and microsatellite genetic markers.

Materials and Methods

Fish samples and DNA extraction

In 2010 a few dozen Thai-Chitralada fish were introduced to Israel from Thailand, kept in isolation and then transferred to the Dor Research Station. DNA was extracted from the caudal fin of 50 Thai-Chitralada individuals randomly selected from the introduced stock. Hundreds of random samples for each of six different tilapia species (*O. aureus*, *O. niloticus*, *O. mossambicus*, *O. urolepis hornorum*, *Sarotherodon galilaeus* and *Tilapia zillii*) during the last 12 years were collected from different regions and stocks in Israel and were subjected to DNA extraction using MasterPure DNA Purification Kit (Madison, USA).

DNA barcoding and microsatellites analysis

PCR for 19 Thai-Chitralada fish (9 males and 10 females) and samples of different tilapia species was performed for the mitochondrial *COI* and D-loop region following Shirak et al. 2016 and Nyingi and Agnèse, 2007, respectively. The resulting sizes of PCR products were 615 bp for *COI* and 410-417 bp for D-loop region. The use of two different mitochondrial regions is aimed to expand the sequence comparison, and test for concordance of the results from both analyses. Sequence trace files for the mitochondrial sites were assembled using the GAP4 program (Staden et al., 2000). Homology of *COI* sequences within the strain was determined if < 1% nucleotide differences was evident from the consensus sequence of the strain (Kesk n and Atar 2013; Shirak et al., 2009). A phylogenetic tree was constructed based on *COI* and D-loop sequences of Thai-Chitralada individuals and the consensus sequences representing the six tilapia species, using the default model recommended by the FISH-BOL bioinformatic tool of MEGA version 10 (Kumar et al., 2018). Microsatellites BYL018, UNH168 (Shirak et al., 2018) and G7A (forward primer: AGTGCTCGGGGATAAAGTCA and reverse primer:

CCACTGAAAGCAGCTTCGTA) on linkage groups 1, 3, and 7, respectively, were genotyped following Shirak et al. (2009) for 50 samples of Thai-Chitralada and tens of samples of *O. aureus*, *O. niloticus* (Egypt and Ghana), and an unknown species that was previously defined as *O. mossambicus*.

Results

Characterization of the main *Oreochromis* strains

BOLD public sequences for COI defined as *O. aureus* (58) and *O. mossambicus* (112) showed 95 and 82% homology of < 1% nucleotide differences, respectively, with the consensus sequences of their species. As for *O. niloticus*, 131 out of 190 COI sequences (69%) clustered with the consensus sequence of this species implying high rates of erroneous identification for the remainder sequences. All consensus sequences for *O. aureus* and *O. niloticus* were similar or identical to those of the strains maintained in Israel. However, our previously sequences reported to be *O. mossambicus* were rejected by BOLD, as they were different from its consensus sequence (Shirak et al., 2009).

DNA barcoding

COI sequences of 19 Chitralada fish were classified by cluster analysis into three groups comprising 6, 7, and 6 individuals (Figure 1). The three groups were similar with < 1% nucleotide differences to the consensus sequences of *O. aureus*, *O. niloticus*, and *O. mossambicus*. Results of the D-loop region analysis were consistent with those of the COI results (data not shown).

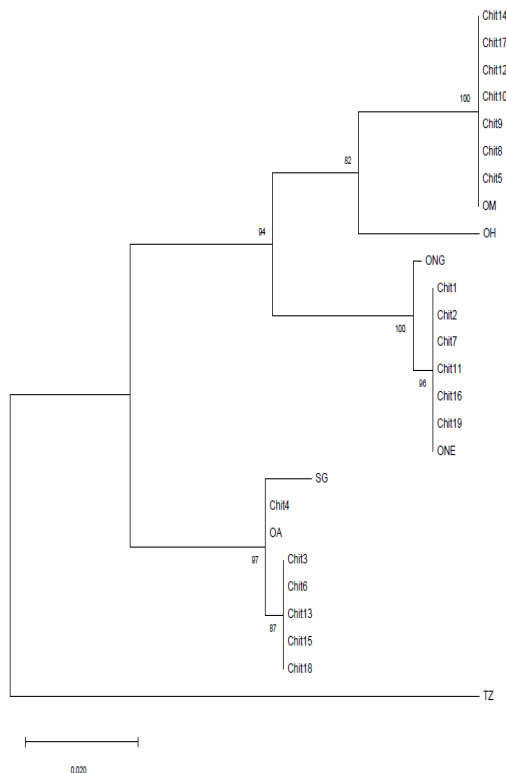


Figure 1: Phylogenetic tree based on COI sequences of 19 Thai-Chitralada samples (Chit1-19) and consensus sequences of *O. niloticus* from Ghana (ONG) and Egypt (ONE), *O. aureus* (OA), *S. galilaeus* (SG), *O. urolepis hornorum* (OH), *T. zillii* (TZ) and *O. mossambicus* (OM). Distances were calculated using the model of Kimura 2-parameter. The tree was generated by MEGAX using neighbor-joining method and bootstrap analysis with 500 replicates. Numbers on the tree junctions indicate the percentage of trees that correspond to the consensus bootstrap tree. Scale on the X-axis (0.020) represents rate of nucleotide substitutions.

Microsatellites analysis

In Figure 2 different *Oreochromis* species are characterized for their allele distribution for three microsatellite markers: BYL018, UNH168, and G7A. A specific interval of alleles for each marker was evident for each species. The allele ranges for UNH168 and G7A exclude *O. niloticus* (Ghana) as a potential contributor to the Thai-Chitralada hybrid. The results of BYL018 revealed that one Thai-Chitralada allele (238), could have originated from *O. aureus*, and four other alleles (246, 252, 254 and 268) could have originated from *O. niloticus*, but an additional allele (286) may have

originated from other species. Similarly, two Thai-Chitralada alleles of UNH168 may have originated from *O. aureus* (148 and 150) and two other alleles (160 and 168) from Egyptian *O. niloticus*, while two other alleles (136 and 144) may have originated from additional species. The results of G7A confirm that Thai-Chitralada actual alleles could have originated from *O. aureus* and Egyptian *O. niloticus*. *O. mossambicus* samples in Israel are not available, and thus were not analyzed for these markers.

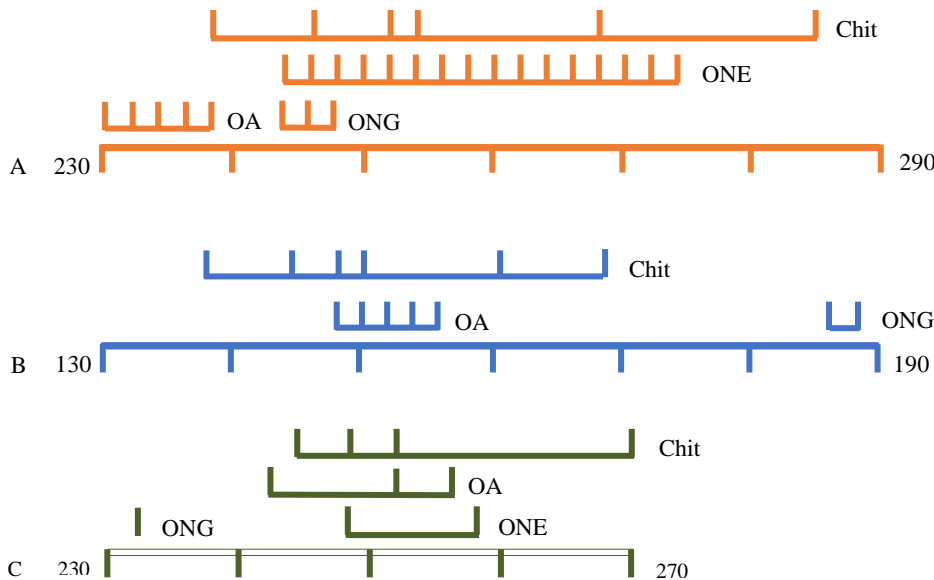


Figure 2: Microsatellite alleles for markers BYL018 (A, in orange), UNH168 (B in blue) and G7A (C in brown) for 50 Thai-Chitralada (Chit) samples and tens of random samples of *O. niloticus* from Ghana (ONG), *O. niloticus* from Egypt (ONE) and *O. aureus* (OA). For each marker the upward grids represent actual alleles. The bottom line with downward grids represents a ladder marking 10 bp intervals.

Discussion

COI was considered the best candidate for a universal barcode for animal life based on the availability of robust universal primers for this sequence, its relatively high rate of molecular evolution, and lack of insertions or deletion mutations relative to ribosomal sequences such as 12S and 16S (Hebert et al., 2003). However, mitochondrial barcode such as *COI* is unlikely to be representative of nuclear divergence and a genetic divergence estimate taken from just one part of the genome does not produce an accurate representation of organismal divergence (i.e. speciation) (Humphries and Winker, 2011). In the current study we employed both mitochondrial DNA barcoding and genomic based microsatellites to study the origin of Thai-Chitralada Tilapia.

Thai-Chitralada, possessing desirable traits for aquaculture, was considered to be an *O. niloticus* strain (Macaranas et al., 1997; Tuan et al., 1999; Mair et al., 2004; Tsadik and Bart 2007; Lago et al., 2017). However, our data based on mitochondrial and genomic analyses demonstrate that at least *O. aureus*, *O. niloticus* (Egypt), and *O. mossambicus* contributed to its formation. Furthermore, D-loop sequences of Thai-Chitralada were identical to a haplotype of *O. mossambicus* collected from Olifants River (Limpopo basin) in South Africa (D'Amato et al., 2007), and homologous with < 1% nucleotide differences to other haplotypes collected in Mozambique (Nagl et al., 2001; Firmat et al., 2013). Comparison of *COI* sequences to BOLD showed that the Israeli stock that originated from Natal (South Africa) was erroneously defined as "*O. mossambicus*". Absence of *O. mossambicus* in Israel excludes the possibility of local admixture of Thai-Chitralada with this species in Israel. Furthermore, although *O. niloticus* from Ghana is prevalent in Israel, *COI* and D-loop sequences of this strain were not evident in Thai-Chitralada, supporting the hypothesis that admixture of Thai-Chitralada occurred before its introduction to Israel along its route and introduction from Egypt to Thailand via Japan. A wider range of sex ratio between different families in the Thai-Chitralada strain than that seen in purebred strains of *O. niloticus*, was noted thus strengthening our

hypothesis (Tuan et al. 1999). This may reflect the diverse mechanisms of sex determination that were implemented in Thai-Chitralada by the different species.

The three species that contributed to the formation of Thai-Chitralada represent diverse geographic regions harboring species from the southern parts of Africa (Mozambique, Zimbabwe, and South Africa, *O. mossambicus*), Northern Africa, and the Middle East (Egypt, *O. niloticus* and *O. aureus*). This diversity may contribute to desired traits in aquaculture. For example, inherited cold tolerance is related to native species in northern areas like *O. aureus* (Smitherman and Khather, 1988; Behrends et al., 1990), whereas tolerance to high salinity has been associated with southern *Oreochromis* species such as *O. mossambicus* (Stickney, 1986; Kamal and Mair, 2005; Firmat et al., 2013). Thai-Chitralada may also receive these genetic capabilities through heterosis (Cai et al., 2004; Moreira et al., 2005; Neves et al., 2008; Pongthana et al., 2010). Several selective breeding programs demonstrated high potential of tilapia breeding and significantly higher growth rate of improved strains than purebred local varieties (Ansah et al., 2014). There are significant changes in conditions for raising tilapia in different geographical regions in Israel mainly due to climate and water characteristics. Thai-Chitralada, because of its diverse genetic background and desirable traits, can be used as a genetic resource for selection and adaptation to different geographical regions. Alleles for resistance to cold, originating from *O. aureus*, may be selected in northern regions like the Galilee and Golan heights, whereas alleles for water salinity originating from *O. mossambicus* may be selected in the southern region of the Negev and Arava. Likewise, alleles for enhanced growth rate originating from *O. niloticus* may be selected in seasonal ponds. Markers assisted selection can be used to enhance breeding for these goals.

Acknowledgements

This work was supported by the Fish Breeders Organization, Israel (grant number 07-2018).

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